Exhibit 8

Exhibit 8

Influence of Formulation Vehicle on Metronomic Taxane Chemotherapy: Albumin-Bound versus Cremophor EL–Based Paclitaxel

Sylvia S.W. Ng,[1] Alex Sparreboom,[2] Yuval Shaked,[1] Christina Lee,[1] Shan Man,[1] Neil Desai,[3] Patrick Soon-Shiong,[3] William D. Figg,[2] and Robert S. Kerbel[1]

Abstract

Purpose: Low-dose metronomic chemotherapy treatments, especially when combined with 'dedicated' antiangiogenic agents, can induce significant antitumor activity without serious toxicity in various preclinical models. It remains unclear, however, whether some cytotoxic drugs are better suited for metronomic regimens than others. Paclitaxel appears to be a strong candidate for metronomic chemotherapy given its ability to inhibit endothelial cell functions relevant to angiogenesis in vitro at extraordinarily low concentrations and broad-spectrum antitumor activity. Clinically relevant concentrations of the formulation vehicle cremophor EL in Taxol, however, were previously reported to nullify the antiangiogenic effect of paclitaxel, the result of which would hamper its usefulness in metronomic regimens. We hypothesized that ABI-007, a cremophor EL–free, albumin-bound, 130-nm form of paclitaxel, could potentially alleviate this problem.

Experimental Design: The antiangiogenic activity of ABI-007 was assessed by multiple in vitro assays. The in vivo optimal dose of ABI-007 for metronomic chemotherapy was determined by measuring circulating endothelial progenitors in peripheral blood. The antitumor effects of metronomic and maximum tolerated dose ABI-007 and Taxol were then evaluated and compared in severe combined immunodeficient mice bearing human MDA-MD-231 breast cancer and PC3 prostate cancer xenografts.

Results: ABI-007 significantly inhibited rat aortic microvessel outgrowth, human endothelial cell proliferation, and tube formation. The optimal metronomic dose of ABI-007 was determined to be between 3 and 10 mg/kg. Metronomic ABI-007 but not Taxol, significantly suppressed tumor growth in both xenograft models. Furthermore, the antitumor effect of minimally toxic metronomic ABI-007 approximated that of the maximum tolerated dose of Taxol.

Conclusions: Our results underscore the influence of formulation vehicles on the selection of cytotoxic drugs for metronomic chemotherapy.

An alternative dosing regimen to pulsatile maximum tolerated dose (MTD) or "dose dense" and dose-intensive chemotherapy is "metronomic chemotherapy": the frequent administration of such drugs at close regular intervals with no prolonged breaks over long periods of time (1). The reduced toxicity and comparable or even increased efficacy of metronomic regimens compared with some MTD counterparts have been shown in a number of preclinical models (2, 3). In addition, metronomic chemotherapy regimens are particularly well suited for long-term combination with relatively non–toxic-targeted biological therapeutics especially antiangiogenic drugs (4, 5), sometimes being used after an initial short course of MTD chemotherapy (i.e., "chemo-switching" protocols; refs. 4, 6). Some phase II clinical trials have been reported with encouraging results, both in terms of antitumor efficacy and reduced toxicity, although such results need to be validated in larger randomized phase II or III trials (7–9). These trials have involved combinations of two metronomically given drugs [e.g., cyclophosphamide and methotrexate (7) or cyclophosphamide and etoposide (8)] given orally on a daily basis, or similar protocols in combination with a drug such as bevacizumab (Avastin), the anti-vascular endothelial growth factor antibody (9).

Virtually every class of chemotherapeutic drug has been reported to have antiangiogenic properties (10), which in many cases can be amplified by metronomic dosing schedules (1).

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Received 12/16/05; revised 4/21/06; accepted 5/10/06.

Grant support: National Cancer Institute of Canada (NCIC), Canadian Institutes of Health Research (R.S. Kerbel) and Terry Fox Research Grant for New Investigators funded through the NCIC.

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Note: S.S.W. Ng and Y. Shaked are recipients of postdoctoral fellowships from the Canadian Institutes of Health Research.

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doi:10.1158/1078-0432.CCR-05-2762

However, the selection of chemotherapeutic drugs for metronomic regimens remains somewhat arbitrary. It is not clear whether some agents or classes of agents are better suited for metronomic chemotherapy than others. Taxanes, such as paclitaxel, would seem to be excellent candidates based on the finding that ultra low (e.g., picomolar) concentrations of paclitaxel can selectively inhibit endothelial functions relevant to angiogenesis, or even kill such cells (11–15). In addition, long-term metronomic chemotherapy using microtubule-inhibiting taxanes as opposed to mutagenic and hence potentially carcinogenic DNA damaging drugs, such as alkylating agents, could also be an advantage, especially in patients receiving adjuvant metronomic therapy regimens over long periods of time for early-stage disease (16). Furthermore, taxane metronomic chemotherapy may be useful to combine with metronomic chemotherapy using another class of drug, such as anti-metabolites (e.g., UFT, the oral 5-fluorouracil prodrug; ref. 16). For example, we have recently reported that a concurrent combination of daily oral low-dose UFT and cyclophosphamide can successfully control highly advanced visceral metastases of human breast cancer in immunodeficient mice, whereas UFT or cyclophosphamide used alone could not (17). However, clinically relevant concentrations of the formulation vehicle cremophor EL in Taxol were previously reported to nullify the antiangiogenic activity of paclitaxel, suggesting that this agent or other anticancer drugs formulated in cremophor EL and other commonly used solubilization vehicles, such as polysorbate 80 (Tween 80), may need to be used at much higher doses than anticipated to achieve effective metronomic chemotherapy (18). As such, the advantage of reduced acute serious side effects associated with low-dose paclitaxel regimens versus conventional MTD paclitaxel may be compromised. Clearly, the presence of cremophor EL in Taxol hampers the use of paclitaxel in metronomic chemotherapy. This may explain, for example, the results of Klement et al. (19), in which metronomic Taxol on its own had little obvious effects on transplanted primary human breast tumors in several models.

ABI-007 (Abraxane), a novel cremophor EL–free, albumin-bound, 130-nm form of paclitaxel, was developed to retain the therapeutic benefits of paclitaxel but eliminate cremophor EL–associated toxicities in the Taxol formulation. Several clinical trials have shown the improved pharmacokinetic and toxicity profiles as well as therapeutic efficacy of ABI-007 over Taxol in MTD regimens (20–24). We hypothesized that the cremophor EL–free nature of ABI-007 may render paclitaxel-based metronomic chemotherapy feasible. With this in mind, the primary objective of this study was to evaluate the therapeutic potential of paclitaxel-based metronomic regimens using ABI-007.

Materials and Methods

Drugs. Taxol injection containing paclitaxel at 6 mg/mL in a mixture of cremophor EL and ethanol USP (1:1 v/v; Bristol-Myers Squibb Canada, Montreal, Canada) was purchased from the local hospital pharmacy. ABI-007 was obtained from American BioScience (Santa Monica, CA).

Rat aortic ring assay. Twelve-well tissue culture plates were coated with 250 μL of Matrigel (Collaborative Biomedical Products, Bedford, MA) and allowed to gel for 30 minutes at 37°C and 5% CO_2. Thoracic aortas were excised from 8- to 10-week-old male Sprague-Dawley rats. Following removal of fibroadipose tissues, the aortas were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells, and covered with an additional 250 μL of Matrigel. After the second layer of Matrigel had set, the rings were covered with EGM-II and incubated overnight at 37°C and 5% CO_2. EGM-II consists of endothelial cell basal medium (EBM-II; Cambrex, Walkersville, MD) plus endothelial cell growth factors provided as the EGM-II Bulletkit (Cambrex). The culture medium was subsequently changed to EBM-II supplemented with 2% fetal bovine serum, 0.25 μg/mL amphotericin B, and 10 μg/mL gentamicin. Aortic rings were treated with EBM-II containing the vehicle (0.9% saline/albumin), carboxyamidotriazole (12 μg/mL), or ABI-007 (0.05-10 nmol/L paclitaxel) for 4 days and photographed on the fifth day using a ×2.5 objective. Carboxyamidotriazole, a known antiangiogenic agent, was used at higher than clinically achievable concentration as positive control (25). Experiments were repeated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop 6.0.

Endothelial cell proliferation assay. Human umbilical vein endothelial cells (HUVEC; Cambrex) were maintained in EGM-II at 37°C and 5% CO_2. HUVECs were seeded onto 12-well plates at a density of 30,000 per well and allowed to attach overnight. The culture medium was then aspirated, and fresh culture medium containing either the vehicle (0.9% saline/albumin), or ABI-007 (0.05-10 nmol/L paclitaxel) was added to each well. After 48 hours, cells were trypsinized and counted with a Coulter Z1 counter (Coulter Corp., Hialeah, FL). All experiments were repeated thrice.

Endothelial cell tube formation assay. Eight-well slide chambers were coated with 150 μL of Matrigel and allowed to gel at 37°C and 5% CO_2 for 30 minutes. HUVECs were then seeded at 30,000 per well in EGM-II containing either the vehicle (0.9% saline/albumin) or ABI-007 (0.05-10 nmol/L paclitaxel) and incubated at 37°C and 5% CO_2 for 16 hours. After incubation, slides were washed in PBS, fixed in 100% methanol for 10 seconds, and stained with DiffQuick solution II (Dade Behring, Inc., Newark, DE) for 2 minutes. To analyze tube formation, each well was digitally photographed using a ×2.5 objective. A threshold level was set to mask the stained tubes. The corresponding area was measured as the number of pixels using MetaMorph software (Universal Imaging, Downingtown, PA). Experiments were repeated thrice.

Determination of the* in vivo *optimal biological dose of ABI-007 by measuring circulating endothelial cells and circulating endothelial progenitors. Six- to 8-week-old female BALB/cJ mice were randomized into the following eight groups (n = 5 each): untreated, treated with i.p. bolus injections of either the drug vehicle (0.9% saline/albumin), or ABI-007 at 1, 3, 6, 10, 15, or 30 mg/kg paclitaxel daily for 7 days. At the end of the treatment period, blood samples were drawn by cardiac puncture and collected in EDTA-containing vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Circulating endothelial cells (CEC) and circulating endothelial progenitors (CEP) were enumerated using four-color flow cytometry as previously described (3, 26). Monoclonal antibodies specific for CD45 were used to exclude $CD45^+$ hematopoietic cells. CECs and their CEP subset were depicted using the murine endothelial markers fetal liver kinase 1/vascular endothelial growth factor receptor 2, CD13, and CD117 (BD PharMingen, San Diego, CA). Nuclear staining (Procount; BD Biosciences, San Jose, CA) was done to exclude the possibility of platelets or cellular debris interfering with the accuracy of CEC and CEP enumeration (27, 28). After red cell lysis, cell suspensions were evaluated by a FACSCalibur (BD Biosciences) using analysis gates designed to exclude dead cells, platelets, and debris. At least 100,000 events per sample were obtained to analyze the percentage of CECs and CEPs. The absolute number of CECs and CEPs was then calculated as the percentage of the events collected in the CEC and CEP enumeration gates multiplied by the total white

cell count. Percentages of stained cells were determined and compared with the appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining. 7-Amino-actinomycin D was used to enumerate viable versus apoptotic and dead cells (29).

Human tumor xenograft therapy studies. Human prostate cancer cell line PC3 and human breast cancer cell line MBA-MD-231 were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). All animal experiments were done in accordance with institutional guidelines for animal welfare. PC3 (5×10^6 cells) were injected s.c. into 6- to 8-week-old male severe combined immunodeficient mice, whereas MDA-MB-231 (2×10^6 cells) were implanted orthotopically into the mammary fat pad of female severe combined immunodeficient mice. When the primary tumor volume reached ~150 to 200 mm^3, animals were randomized into eight groups (n = 5-10 per group). Each group was treated with either 0.9% saline/albumin vehicle control, cremophor EL vehicle control, metronomic Taxol (1.3 mg/kg, i.p., qd), metronomic ABI-007 (3, 6, or 10 mg/kg paclitaxel, i.p., qd), MTD Taxol (13 mg/kg, i.v., qd×5, 1 cycle), or MTD ABI-007 (30 mg/kg paclitaxel, i.v., qd×5, 1 cycle). Perpendicular tumor diameters were measured with a caliper once a week, and their volumes were calculated using the formula $\pi/6 \times a \times b^2$, where a is the longest dimension of the tumor, and b is the width. At the end of the treatment period, blood samples were drawn by cardiac puncture from mice in all groups and collected in EDTA-containing vacutainer tubes (Becton Dickinson). CECs and CEPs were enumerated as described above. Tumors were harvested, snap frozen in optimum cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) in liquid nitrogen and subsequently processed for immunofluorescence staining.

Detection and quantification of intratumoral microvessel density. Five-micrometer-thick sections obtained from each frozen tumor were stained with H&E for histologic examination. For detection of microvessels, sections were stained with a rat anti-mouse CD31/platelet/endothelial cell adhesion molecule 1 antibody (1:1,000; BD PharMingen) followed by a Texas Red–conjugated goat anti-rat secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). A single microvessel was defined as a discrete cluster or single cell stained positive for CD31/platelet/endothelial cell adhesion molecule 1, and the presence of a lumen was not required for scoring as a microvessel. The microvessel density for each tumor was expressed as the average count of the three most densely stained fields identified with a ×20 objective on a Zeiss AxioVision 3.0 fluorescence microscopic imaging system. Four to five different tumors per each vehicle control or treatment group were analyzed.

In vivo *angiogenesis evaluation.* The Matrigel plug perfusion assay was done with minor modifications as previously described (4). Briefly, 0.5 mL Matrigel supplemented with 500 ng/mL of basic fibroblast growth factor (R&D Systems, Inc., Minneapolis, MN) was injected s.c. on day 0 into the flanks of 10-week-old female BALB/cJ mice. On day 3, animals were randomly assigned to eight groups (n = 5 each). Each group was treated with either 0.9% saline/albumin vehicle control, cremophor EL vehicle control, metronomic Taxol (1.3 mg/kg, i.p., qd), metronomic ABI-007 (3, 6, or 10 mg/kg paclitaxel, i.p., qd), MTD Taxol (13 mg/kg, i.v., qd×5), or MTD ABI-007 (30 mg/kg paclitaxel, i.v., qdx5). As a negative control, five additional female BALB/cJ mice of similar age were injected with Matrigel alone. On day 10, all animals were injected i.v. with 0.2 mL of 25 mg/mL FITC-dextran (Sigma, St. Louis, MO). Plasma samples were subsequently collected. Matrigel plugs were removed, incubated with Dispase (Collaborative Biomedical Products, Bedford, MA) overnight at 37°C, and then homogenized. Fluorescence readings were obtained using a FL600 fluorescence plate reader (Biotech Instruments, Winooski, VT). Angiogenic response was expressed as the ratio of Matrigel plug fluorescence to plasma fluorescence.

Statistics. All results were presented as mean ± SE. Comparisons were made with one-way ANOVA followed by Student Newman-Keuls or Dunnett's test with $P < 0.05$ as the criterion for statistical significance. Correlation between viable CEP levels in peripheral blood and intratumoral microvessel density in the human tumor xenografts studies was examined using the nonparametric Spearman test with the level of significance set at $P < 0.05$.

Results

Rat aortic angiogenesis, HUVEC proliferation, and tube formation in response to ABI-007. We first asked whether the antiangiogenic effects of paclitaxel can be effectively delivered by the cremophor EL–free ABI-007 *in vitro*. As shown in Fig. 1A, ABI-007 suppressed aortic microvessel outgrowth in a

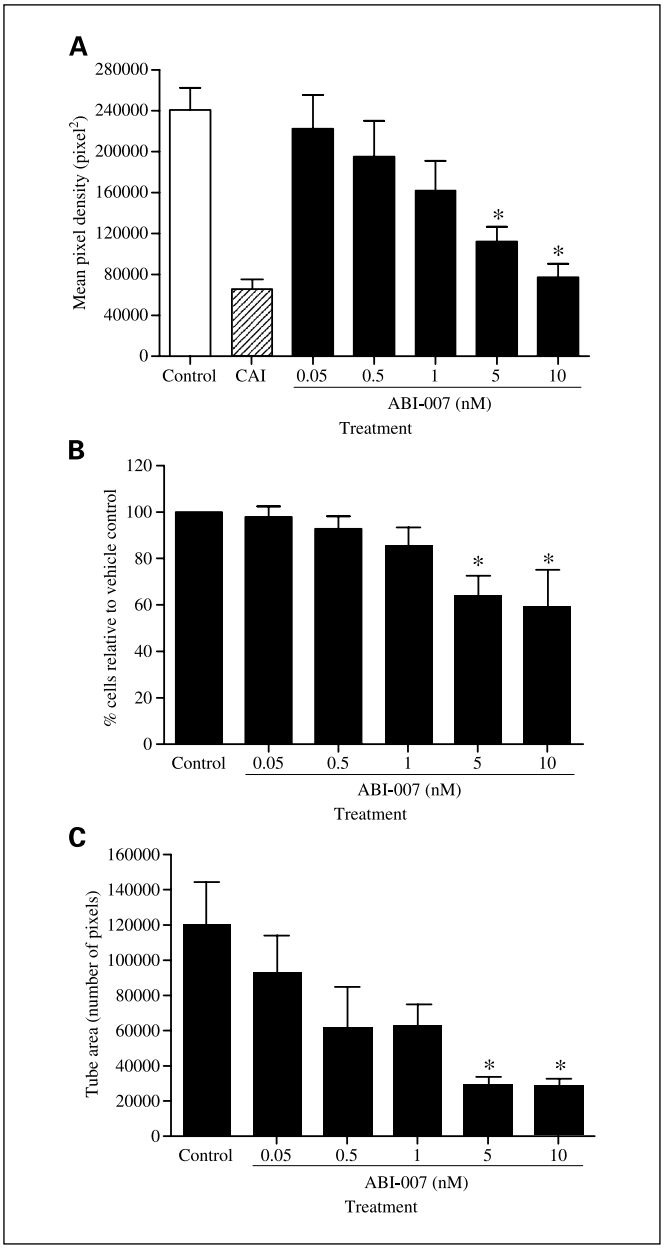

Fig. 1. Effects of ABI-007 on rat aortic ring angiogenesis (*A*), HUVEC proliferation (*B*), and tube formation (*C*). Columns, mean; bars, SE. *, $P < 0.05$, significantly different from the vehicle control. CAI, carboxyamidotriazole.

concentration-dependent manner relative to the vehicle control, reaching statistical significance at 5 nmol/L (53% inhibition) and 10 nmol/L (68% inhibition). The amount of albumin present at each concentration of ABI-007 alone did not inhibit angiogenesis (data not shown).

HUVEC proliferation was significantly inhibited by ABI-007 at 5 and 10 nmol/L by 36% and 41%, respectively. ABI-007 also blocked tube formation by 76% at both 5 and 10 nmol/L (Fig. 1B and C).

In vivo *optimal biological dose for metronomic ABI-007.* Next, the optimal dose of ABI-007 to be used in metrnomic chemotherapy was determined. Enumeration of viable CEPs can be used as a surrogate pharmacodynamic marker to establish the optimal biological dose for targeted antiangiogenic drugs (26) and metronomic chemotherapy (30). Figure 2 shows that ABI-007 given i.p. daily for 7 days at 3 and 10 to 30 mg/kg significantly decreased CEP levels in non–tumor-bearing BALB/cJ mice. However, ABI-007 at 10 to 30 mg/kg was associated with a significant reduction of white blood cell count indicative of toxicity (data not shown). Although the reduction of CEP levels by ABI-007 at 6 mg/kg did not reach statistical significance, decrease in white blood cell count was not evident. We, therefore, concluded that the *in vivo* optimal biological dose for metronomic ABI-007 was between 3 and 10 mg/kg. In a preliminary study, metronomic Taxol at 1.3, 3, 6, or 13 mg/kg given i.p. (qdx7) did not significantly reduce viable CEP levels, whereas metronomic Taxol at 30 mg/kg or higher resulted in severe toxicity and eventually mortality in mice (data not shown). It was previously reported that the i.p. administration of Taxol at doses commonly used in the clinic resulted in entrapment of paclitaxel in cremophor EL micelles in the peritoneal cavity and, consequently, insignificant plasma paclitaxel concentration (31). This would explain why doses of metronomic Taxol (1.3, 3, 6, and 13 mg/kg) that did not cause death failed to change viable CEP levels. In this case, the i.p. administration of metronomic Taxol at 1.3 mg/kg would not be any different from that at 13 mg/kg. We, therefore, selected the lower dose (1.3 mg/kg) to minimize the amount of cremophor EL per paclitaxel administration for subsequent experiments.

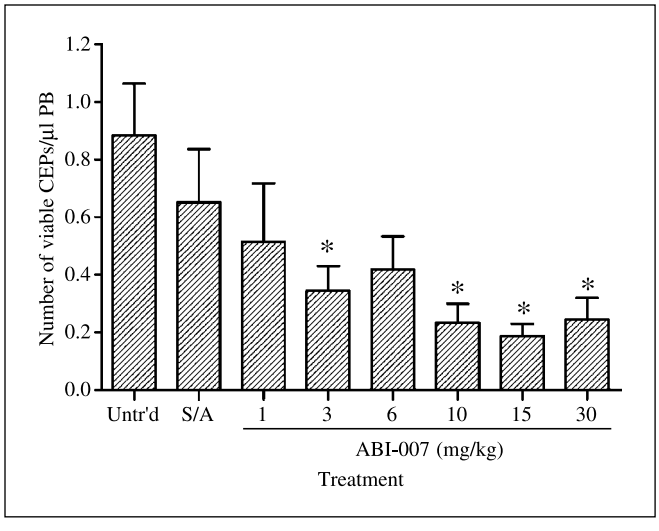

Fig. 2. Determination of optimal biologic dose of ABI-007 for metronomic dosing. Levels of viable CEPs in peripheral blood of BALB/cJ mice in response to escalating doses of ABI-007. Untr'd, untreated control; S/A, saline/albumin vehicle control. Columns, mean; bars, SE. *, $P < 0.05$, significantly different from the untreated control.

Antitumor effects of metronomic and MTD ABI-007 versus Taxol. Metronomic ABI-007 (3, 6, and 10 mg/kg) but not Taxol (1.3 mg/kg) given i.p. daily for 4 weeks significantly inhibited growth of both MDA-MB-231 and PC3 tumors (Fig. 3A and B). Neither ABI-007 nor Taxol given metronomically induced weight loss in mice (Fig. 3C and D). Although MTD ABI-007 (30 mg/kg) suppressed tumor growth more effectively than MTD Taxol (13 mg/kg), significant weight loss was noted with the former, indicating toxicity (Fig. 3C and D). In addition, two of five mice treated with MTD ABI-007 displayed signs of paralysis in one limb 6 days after the last dose of drug. The paralysis was transient and resolved within 24 to 48 hours. Interestingly, the antitumor effect of metronomic ABI-007 at 6 mg/kg approximated that of MTD Taxol in the MDA-MB-231 xenograft model (Fig. 3A). Increasing the dose of metronomic ABI-007 to 10 mg/kg did not seem to confer more pronounced tumor growth inhibition (Fig. 3A). In contrast, metronomic ABI-007 elicited greater antitumor response at 10 mg/kg than at 3 and 6 mg/kg in the PC3 xenografts (Fig. 3B).

Viable CEP levels in response to metronomic and MTD ABI-007 versus Taxol. As illustrated in Fig. 4A, metronomic ABI-007 significantly decreased the levels of viable CEPs in a dose-dependent manner in MDA-MB-231 tumor-bearing mice. Viable CEP levels also exhibited a dose-dependent reduction in response to metronomic ABI-007 in PC3 tumor-bearing mice but reaching statistical significance only at 10 mg/kg (Fig. 4B). Although metronomic Taxol seemed to reduce the levels of CEPs slightly in both xenograft models, the reduction did not reach statistical significance (Fig. 4A and B). Both MTD ABI-007 and MTD Taxol significantly lowered viable CEPs compared with their respective vehicle controls in MDA-MB-231 tumor-bearing mice, whereas only MTD Taxol did so in the PC3 tumor-bearing mice (Fig. 4A and B).

Effects of metronomic and MTD ABI-007 and Taxol on intratumoral microvessel density. In MDA-MB-231 tumors, metronomic ABI-007 at 6 and 10 mg/kg as well as MTD ABI-007 seemed to reduce microvessel density slightly although statistical significance was not reached (Fig. 5A). In PC3 tumors, metronomic ABI-007 at 3 and 10 mg/kg seemed to decrease microvessel density but without reaching statistical significance (Fig. 5A). Interestingly, a significant correlation existed between microvessel density and the levels of viable CEPs in the MDA-MB-231 (Fig. 5B; $r = 0.76$, $P = 0.04$) but not in the PC3 (Fig. 5C; $r = -0.071$, $P = 0.88$) model.

In vivo *antiangiogenic activity of metronomic and MTD ABI-007 versus Taxol.* In the Matrigel plug perfusion assay, metronomic ABI-007 at 6 and 10 mg/kg seemed to decrease angiogenesis, although the inhibition did not reach statistical significance (Fig. 6). Angiogenesis seemed to be unaltered by metronomic ABI-007 at 3 mg/kg, MTD ABI-007, MTD, and metronomic Taxol relative to the respective vehicle controls (Fig. 6). These observations were similar to the intratumoral microvessel density results described above.

Discussion

Our results show that metronomic chemotherapy using albumin-bound nanoparticle paclitaxel (ABI-007), but not

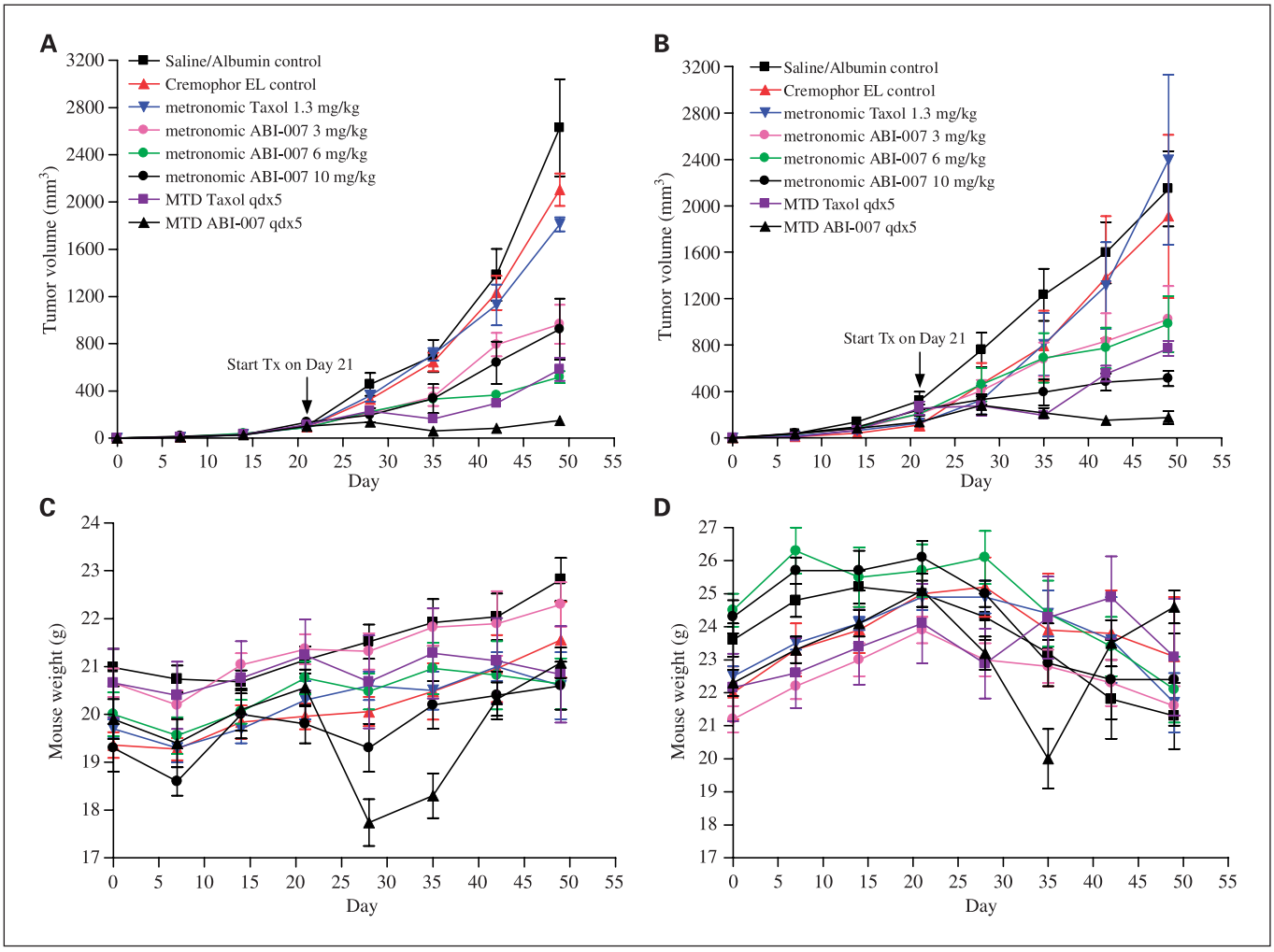

Fig. 3. Effects of ABI-007 and Taxol used in metronomic or MTD regimens on MBA-MD-231 (*A*) and PC3 (*B*) tumor growth and the body weight of MDA-MB-231 (*C*) and PC3 (*D*) tumor-bearing SCID mice. Five to 10 mice were used per control or treatment group. Points, mean; bars, SE.

paclitaxel formulated in cremophor EL (Taxol), exhibits potent *in vivo* antitumor activity. It was previously reported that clinically relevant concentrations of formulation vehicles, such as cremophor EL, nullify the *in vitro* antiangiogenic effect of taxanes (18). Paclitaxel at 4 nmol/L dissolved in DMSO but not in cremophor EL suppressed rat aortic angiogenesis and HUVEC proliferation (18). In the present study, ABI-007 at 5 nmol/L induced responses similar to those elicited by paclitaxel at 4 nmol/L dissolved in DMSO, indicating that the antiangiogenic property of paclitaxel was effectively delivered by cremophor EL–free ABI-007. Desai et al. (32) recently showed that paclitaxel in ABI-007 is actively transported into and across endothelial cells by gp60 (a specific albumin receptor)–mediated caveolar transcytosis, a process that is inhibited by cremophor EL in Taxol.

One cycle of either MTD ABI-007 or MTD Taxol was shown herein to cause marked tumor growth inhibition and transient regression in two different xenograft models (MDA-MB-231 and PC3). However, MTD Taxol–treated tumors rapidly resumed growth 3 weeks after the last dose of drug (day 42), whereas the growth of MTD ABI-007–treated tumors continued to be suppressed. The ability of MTD ABI-007 but not MTD Taxol to significantly reduce viable CEPs in MDA-MB-231 tumor-bearing mice might contribute, at least in part, to the enduring antitumor effect of the former. A higher paclitaxel plasma clearance and a larger volume of distribution for ABI-007 than for Taxol was recently reported in humans (33) as well as more rapid cellular uptake and binding (32), suggesting that paclitaxel in the ABI-007 formulation might be more effective against CEPs than Taxol. Higher intratumoral paclitaxel concentration achieved by the ABI-007 formulation could be another contributing factor (32, 33). We cannot explain why there was a lack of a significant decrease in viable CEPs by MTD ABI-007 in PC3 tumor-bearing mice. The possibility that the s.c. PC3 tumors might be less dependent on recruitment of CEPs for angiogenesis compared with the orthotopic MDA-MB-231 tumors cannot be excluded.

The significant weight loss and transient paralysis associated with MTD ABI-007 treatment and the absence of cremophor EL in the formulation prompted us to explore the feasibility of using ABI-007 in metronomic regimens as a less toxic but still highly effective antitumor alternative, one that would be well suited for long-term combination with other drugs, such as vascular endothelial growth factor–targeted antiangiogenic

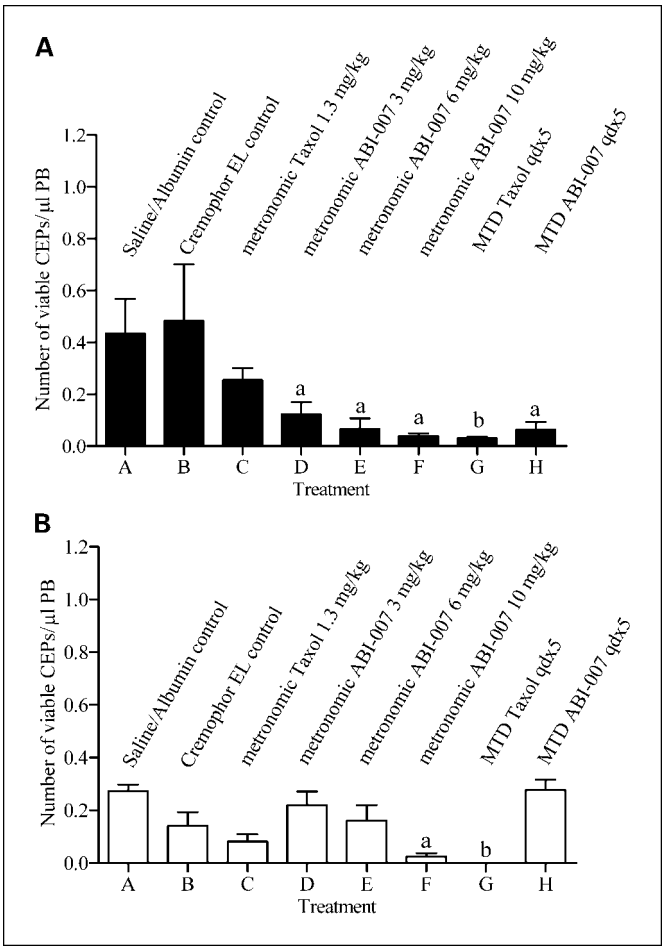

Fig. 4. Changes in the levels of viable CEPs in peripheral blood of MDA-MB231 (*A*) and PC3 (*B*) tumor-bearing severe combined immunodeficient mice after treatment with metronomic or MTD ABI-007 and Taxol. Columns, mean; bars, SE. a, $P < 0.05$, significantly different from saline/albumin vehicle control. b, $P < 0.05$, significantly different from cremophor EL vehicle control.

drugs (4, 6). Naturally, the selection of optimal doses of ABI-007 for such regimen is pivotal to achieving therapeutic efficacy. We have recently shown the potential use of viable CEP levels in peripheral blood as a biomarker to determine the optimal biological doses of antiangiogenic drugs (26) and for metronomic chemotherapy, at least in mice (30). For example, the dose of DC101 (800 μg), a rat monoclonal antibody against mouse vascular endothelial growth factor receptor 2, which induced the lowest levels of viable CEPs also elicited the greatest reduction in tumor volume, whereas dose escalation up to 2,000 μg failed to decrease both variables any further (26). In the current study, metronomic ABI-007 at 3 and 10 to 30 mg/kg significantly reduced viable CEP levels in non–tumor-bearing BALB/cJ mice. However, ABI-007 at 10 to 30 mg/kg was also associated with significant reduction in white blood cell count, indicative of toxicity. We, therefore, deduced that the optimal biological doses for metronomic ABI-007 were between 3 and 10 mg/kg. Indeed, metronomic ABI-007 at 3 to 10 mg/kg given i.p. daily for 4 weeks effectively inhibited MDA-MB-231 and PC3 tumor growth. The observed decrease in tumor volume was accompanied by a dose-dependent reduction in viable CEP levels in both xenograft models. It should also be noted that the total dose of ABI-007 given over 4 weeks in the metronomic regimen, especially in the 10 mg/kg/d treatment group, was substantially higher than that given over 5 days in the MTD regimen (280 versus 150 mg), and yet no significant weight loss was evident in the former. In marked contrast, metronomic Taxol failed to suppress tumor growth or significantly alter viable CEP levels. In all likelihood, this can be attributed to the entrapment of paclitaxel in cremophor micelles in the

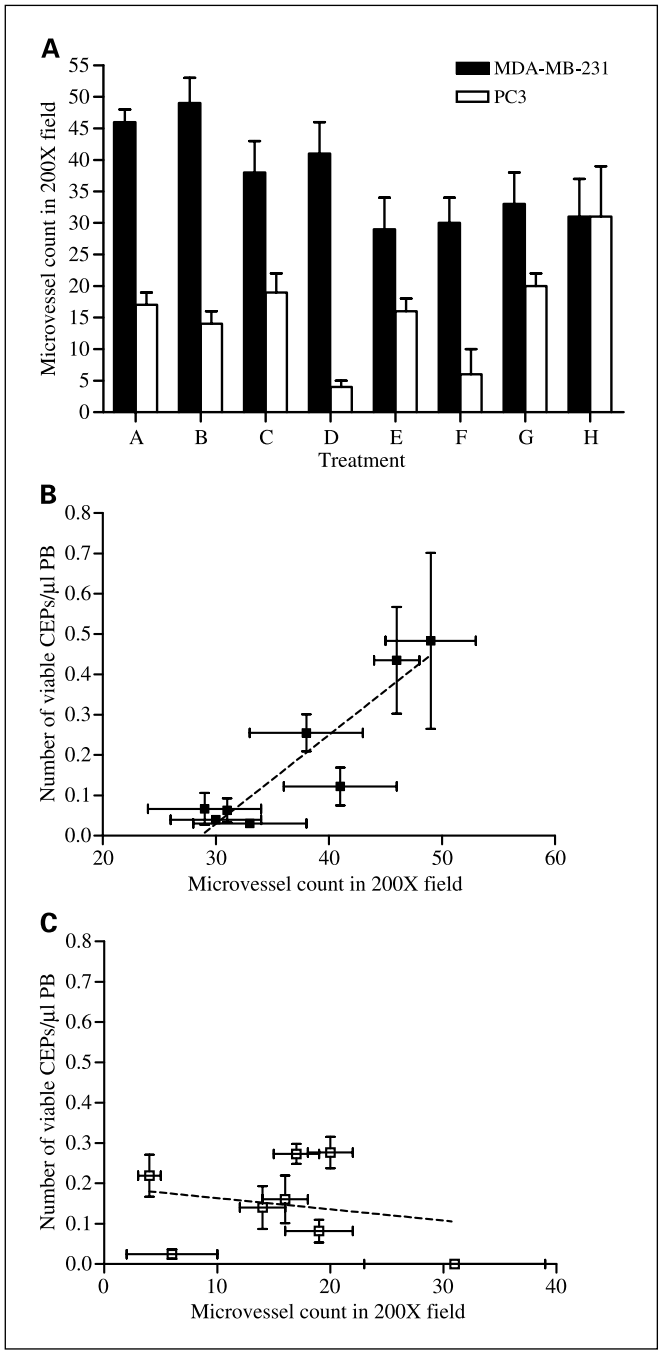

Fig. 5. Intratumoral microvessel density (*A*) of MDA-MB-231 (■) and PC3 (□) xenografts treated with (*A*) saline/albumin control; (*B*) cremophor EL control; (*C*) metronomic 1.3 mg/kg Taxol; (*D, E*, and *F*) metronomic 3, 6, and 10 mg/kg ABI-007, respectively; (*G*) MTD Taxol; and (*H*) MTD ABI-007. Columns, mean; bars, SE. Correlation between intratumoral microvessel density and the number of viable CEPs in peripheral blood in MDA-MB-231 (*B*) and PC3 (*C*) tumor-bearing mice.

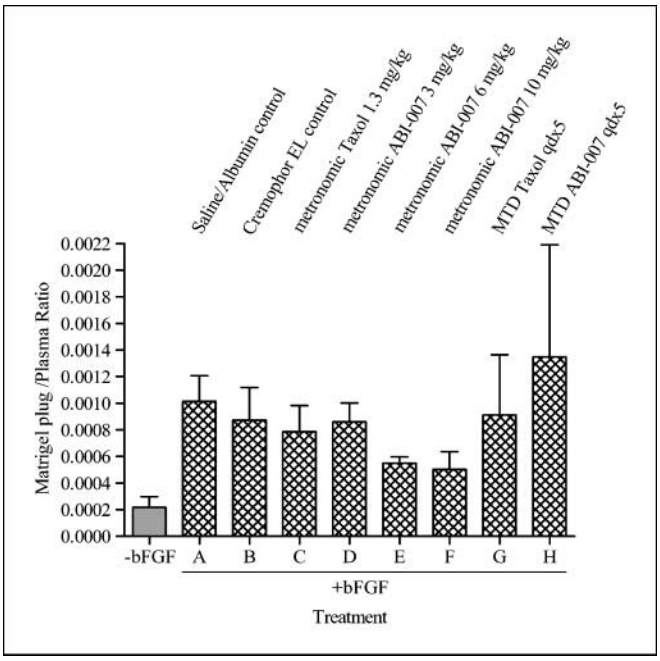

Fig. 6. Effects of metronomic or MTD ABI-007 and Taxol on basic fibroblast growth factor (*bFGF*) – induced angiogenesis in Matrigel plugs injected s.c. into the flanks of BALB/cJ mice. Matrigel implanted without basic fibroblast growth factor (*–bFGF*) served as negative control. Columns, mean; bars, SE.

peritoneal cavity. Gelderblom et al. (31) reported a significant increase in bioavailability and systemic concentrations of i.p. paclitaxel in the absence of cremophor EL in patients. Interestingly, the antitumor effect of metronomic ABI-007 at 6 and 10 mg/kg approximated that of MTD Taxol in MDA-MB-231 and PC3 xenografts, respectively. This observation suggests that metronomic ABI-007 at optimal biological doses may be a more desirable means to administer paclitaxel than MTD Taxol because cremophor EL–associated toxicities are eliminated.

Intratumoral microvessel density seemed to decrease slightly in response to metronomic and MTD ABI-007 but not Taxol, albeit without reaching statistical significance. However, reduction in microvessel density is not always obtained with antiangiogenic agents (34). A significant correlation between microvessel density and the levels of viable CEPs was noted in the MDA-MB-231, but this was not observed in the PC3 xenograft model. The *in vivo* antiangiogenic activity of the various treatment regimens used in the tumor xenograft studies was also evaluated using the Matrigel plug perfusion assay. A trend of decreasing angiogenesis with increasing metronomic ABI-007 doses was evident, whereas MTD Taxol and MTD ABI-007 did not seem to block angiogenesis, although statistical significance was not reached with any treatment. Viable CEP levels may be a more sensitive marker than intratumoral microvessel density or Matrigel plug angiogenesis in the evaluation of response to treatment modalities with an antiangiogenic/antivasculogenic component. Although the mechanistic basis of metronomic chemotherapy is thought to be primarily antiangiogenic (2–4, 30), recent evidence showed that long-term metronomic chemotherapy in mice using cyclophosphamide can also stimulate immune responses by augmenting memory T cells and depleting both regulatory and suppressor T cells (35). We cannot exclude the possibility that metronomic chemotherapy using paclitaxel may also induce similar immunomodulatory responses. It is becoming increasingly clear that metronomic chemotherapy regimens exert multiple effects in addition to angiogenesis inhibition.

In summary, we have shown that paclitaxel in the absence of cremophor EL is a viable and effective drug for metronomic chemotherapy. The therapeutic potential of metronomic ABI-007 given alone or in combination with other anticancer and/or antiangiogenic agents warrants investigation in the clinical setting. Our findings especially underscore the importance of selecting an optimal formulation strategy for cytotoxic drugs (and other investigational agents) to be used in metronomic chemotherapy regimens. Clearly, the potential of the nanoparticle albumin-bound technology extends beyond paclitaxel. For instance, Nab-028, the novel taxane ABI-028 formulated as albumin-bound nanoparticles, showed improved efficacy and lower toxicity than ABI-028 formulated in Tween 80 (36). 17-(Allylamino)-17-demethoxygeldanamycin, a hydrophobic drug that inhibits its HSP90 and shows poor tolerability when solubilized in a DMSO-egg lecithin vehicle, was successfully prepared as an albumin-bound nanoparticle formulation suitable for i.v. administration (37). Finally, with respect to the use of taxanes in metronomic chemotherapy regimens, a comparison of nanoparticle-based formulations, such as ABI-007, with orally bioavailable taxanes (e.g., BMS-275183) would seem timely because our results suggest that metronomic chemotherapy with low-dose, cremophor EL–free taxanes should be feasible and effective.

Acknowledgments

We thank Cassandra Cheng for her excellent secretarial assistance.

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VOLUME 29 · NUMBER 34 · DECEMBER 1 2011

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Gemcitabine Plus *nab*-Paclitaxel Is an Active Regimen in Patients With Advanced Pancreatic Cancer: A Phase I/II Trial

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Submitted April 14, 2011; accepted July 8, 2011; published online ahead of print at www.jco.org on October 3, 2011.

Sponsored by Abraxis BioScience, Los Angeles, CA, a wholly owned subsidiary of Celgene, Summit, NJ. The preclinical studies were supported in part by National Institutes of Health Grants No. CA116554 and CA129963 (M.H.) and CA113669 and CA134767 (A.M.) and by a Stand Up To Cancer-American Association for Cancer Research Dream Team Translational Cancer Research Grant No. SU2C-AACR-DT0509.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Clinical Trials repository link available on JCO.org.

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0732-183X/11/2934-4548/$20.00

DOI: 10.1200/JCO.2011.36.5742

ABSTRACT

Purpose
The trial objectives were to identify the maximum-tolerated dose (MTD) of first-line gemcitabine plus *nab*-paclitaxel in metastatic pancreatic adenocarcinoma and to provide efficacy and safety data. Additional objectives were to evaluate positron emission tomography (PET) scan response, secreted protein acidic and rich in cysteine (SPARC), and CA19-9 levels in relation to efficacy. Subsequent preclinical studies investigated the changes involving the pancreatic stroma and drug uptake.

Patients and Methods
Patients with previously untreated advanced pancreatic cancer were treated with 100, 125, or 150 mg/m^2 *nab*-paclitaxel followed by gemcitabine 1,000 mg/m^2 on days 1, 8, and 15 every 28 days. In the preclinical study, mice were implanted with human pancreatic cancers and treated with study agents.

Results
A total of 20, 44, and three patients received *nab*-paclitaxel at 100, 125, and 150 mg/m^2, respectively. The MTD was 1,000 mg/m^2 of gemcitabine plus 125 mg/m^2 of *nab*-paclitaxel once a week for 3 weeks, every 28 days. Dose-limiting toxicities were sepsis and neutropenia. At the MTD, the response rate was 48%, with 12.2 median months of overall survival (OS) and 48% 1-year survival. Improved OS was observed in patients who had a complete metabolic response on [^{18}F]fluorodeoxyglucose PET. Decreases in CA19-9 levels were correlated with increased response rate, progression-free survival, and OS. SPARC in the stroma, but not in the tumor, was correlated with improved survival. In mice with human pancreatic cancer xenografts, *nab*-paclitaxel alone and in combination with gemcitabine depleted the desmoplastic stroma. The intratumoral concentration of gemcitabine was increased by 2.8-fold in mice receiving *nab*-paclitaxel plus gemcitabine versus those receiving gemcitabine alone.

Conclusion
The regimen of *nab*-paclitaxel plus gemcitabine has tolerable adverse effects with substantial antitumor activity, warranting phase III evaluation.

J Clin Oncol 29:4548-4554. © 2011 by American Society of Clinical Oncology

INTRODUCTION

Metastatic pancreatic ductal adenocarcinoma (PDA) is a lethal disease with approximately 6 months of median survival.[1,2] Gemcitabine is the only approved single agent, with a median survival of 5.7 months and 20% 1-year survival.[3] Except for erlotinib, all phase III trials exploring gemcitabine-based combinations have failed to improve overall survival (OS).[4] Nevertheless, a recent meta-analysis of randomized trials revealed a general survival benefit for gemcitabine-based chemotherapies for patients with good performance status.[5] Because of the moderate activity of the current standard gemcitabine and gemcitabine-based regimens,[3-7] improved therapeutic options are greatly needed.

The selection of *nab*-paclitaxel, a 130-nm albumin-bound formulation of paclitaxel particles (Celgene, Summit, NJ), in combination with the standard gemcitabine was based on a molecular profiling of PDA tumor samples,[8] in which secreted protein acidic and rich in cysteine (SPARC), an albumin-binding protein, was noted to be overexpressed. *nab*-Paclitaxel has shown antitumor activity in various advanced cancer types that overexpress SPARC,[9-11] including breast,[12-14] lung,[15,16] and melanoma.[17]

The objectives of this trial were to identify the maximum-tolerated dose (MTD) of gemcitabine

plus *nab*-paclitaxel as first-line therapy in patients with metastatic PDA and to provide efficacy and safety data to permit the planning of a possible pivotal phase III trial. Additional exploratory objectives were to evaluate SPARC and CA19-9 levels and positron emission tomography (PET) scan response in relation to efficacy. Subsequent preclinical studies in human pancreatic cancer xenografts investigated the underlying biology of the substantial clinical activity seen in this phase I/II study.

PATIENTS AND METHODS

Phase I/II Clinical Study

The study was conducted at four centers in the United States in accordance with the Declaration of Helsinki and Good Clinical Practice, Guidelines of the International Conference on Harmonization. Written informed consent was obtained from all patients before entering the study. Eligibility criteria included age ≥ 18 years and histologically or cytologically confirmed metastatic PDA with measurable disease by computed tomography scan as defined by the Response Evaluation Criteria in Solid Tumor (RECIST) version 1.0 guidelines.[18] Patients had no previous treatment for metastatic disease. Prior adjuvant treatment with fluorouracil or gemcitabine administered as a radiation sensitizer during and up to 4 weeks after radiation therapy was allowed. If a patient received adjuvant therapy, tumor recurrence must have occurred ≥ 6 months after the last treatment. Patients had an Eastern Cooperative Oncology Group performance status of 0 or 1 and had adequate hematologic, hepatic, and renal function.

Study Design

This was an open-label phase I/II study. In the phase I portion, the primary end point was to identify the MTD and dose-limiting toxicities (DLTs) of gemcitabine (1,000 mg/m^2) followed by *nab*-paclitaxel (100, 125, or 150 mg/m^2), administered intravenously (IV) on days 1, 8, and 15, every 28 days, using the standard 3 + 3 phase I dose-escalation design.[19] Per protocol, DLTs were treatment-related toxicities during cycle 1 per National Cancer Institute Common Terminology Criteria of Adverse Events version 3.0, including any grade 4 hematologic toxicity; grade 3 thrombocytopenia with hemorrhage; grade ≥ 3 nausea, vomiting, or diarrhea despite prophylaxis; or any grade ≥ 3 treatment-related nonhematologic toxicity, excluding alopecia and fatigue. Dose escalation was stopped when ≥ one of three patients had DLTs, and the dose below was declared the MTD. Patients continued treatment until disease progression or unacceptable toxicity. In the phase II portion, accrual continued at the MTD to ≥ 42 patients to evaluate the efficacy and safety of the combination. This clinical study also evaluated PET scan response, CA19-9, and SPARC levels in relation to antitumor activity.

Assessments

All patients who received at least one dose of study drugs were evaluated for efficacy and safety. Response was assessed by computed tomography scans at baseline and every 4 weeks on day 1 of each cycle (per RECIST v1.0); an initial response (complete [CR] or partial response [PR]) had to be confirmed at least 4 weeks later. Metabolic activity was assessed by [^{18}F]fluorodeoxyglucose (FDG) PET scans at baseline and at 6 and 12 weeks on the basis of the European Organisation for Research and Treatment of Cancer criteria by an independent investigator.[20] Safety was assessed by the incidence of treatment-related adverse events (AEs), according to the National Cancer Institute Common Terminology Criteria of Adverse Events version 3.0, and incidence of patients experiencing dose modifications, dose interruptions, and/or premature discontinuation of study drug. CA19-9 levels were monitored by investigators at every cycle. Archived tumor blocks, if available, were collected for SPARC analysis.

Statistical Methods for Efficacy End Points and Biomarkers

With a total of 44 patients treated at the MTD, there was ≥ 95% power of observing a serious AE that had an incidence of ≥ 7%. The percentage of patients (with 95% CI) who achieved an objective CR or PR using RECIST criteria were summarized using descriptive statistics. Disease control rate was defined as the percentage of patients with CR, PR, and stable disease (SD) ≥ 16 weeks. Progression-free survival (PFS) was defined as the time from first dose of study drug to the start of disease progression or patient death, whichever occurred first. OS was defined as the time from first dose of study drug to patient death. PFS and OS were analyzed using Kaplan-Meier methods.

To assess possible relationships between CA19-9 and efficacy outcomes, the correlation of maximum decrease from baseline in CA19-9 with survival was analyzed. SPARC immunohistochemistry was performed using a monoclonal and a polyclonal antibody and proprietary methodology. Seven tissue components including tumor cells and stromal components such as fibroblast and inflammatory cells were evaluated. For each tissue component and each antibody, three measures were recorded by two board-certified pathologists at a Clinical Laboratory Improvement Amendments laboratory: maximum intensity, percentage of cells at the maximum intensity, and overall score, providing 42 variables. All variables were standardized across patients via z-score transformation and averaged between the two pathologists. For each patient, an average z-score was calculated across variables. On the basis of the average z-scores ≥ or less than 0, patients were classified into a high- or low-SPARC group, respectively. The difference in OS between the low- and high-SPARC groups was assessed by the log-rank test, and a multivariate Cox regression model was used to assess the independent predictive power of SPARC levels. All statistical analyses for SPARC were carried out in R version 2.12.0.[21]

Preclinical Study Methods

The objectives of these preclinical studies were to evaluate tumor progression, potential changes in the pancreatic stroma, and intratumoral drug penetration.

Xenograft Establishment and Treatment

Fresh pancreatic cancer tissues obtained from 11 chemotherapy-naive patients who underwent surgery at the Johns Hopkins (JH) Hospital were propagated as subcutaneous tumors in 6-week-old female athymic nude mice as a live PancXenoBank.[22] Mice with tumor size of ~200 mm^3 were randomly assigned to four treatment groups (seven to 10 tumors/group): (1) control, (2) gemcitabine 100 mg/kg intraperitoneally (IP) on days 1 and 5 weekly for 4 weeks, (3) *nab*-paclitaxel 30 mg/kg/d IV for 5 consecutive days, and (4) gemcitabine plus *nab*-paclitaxel in the preceding regimens for 4 weeks. A response was defined as a more than 50% regression in tumor size. Animals were killed on day 28. The experimental protocol was approved by the Animal Care and Use Committee at JH University.

Immunohistochemistry

Tumors obtained at euthanasia were immediately flash frozen, and a portion of each tumor was kept in 10% formalin for paraffin embedding. The extent of stromal desmoplasia was determined by an immunohistochemistry assay for collagen 1 (1:500; Abcam, Cambridge, MA).[23] Stromal vascularity was assessed using an anti-CD31 antibody (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA).

Quantitative Real-Time PCR

Endothelial cell content was quantified by real-time polymerase chain reaction (qRT-PCR) for murine-specific nestin (mNestin) transcripts.[24] For qRT-PCR, total RNA was isolated (RNeasy Mini Kit, Qiagen, Santa Clarita, CA), followed by cDNA production (SuperScript III First Strand synthesis kit, Invitrogen, Carlsbad, CA). Relative fold expression of mNestin was calculated using the $2^{-\Delta\Delta Ct}$ method.[25]

Gemcitabine Uptake in Tumors

Mice harboring PANC265 xenograft were treated with gemcitabine at 100 mg/kg IP on day 5 or gemcitabine 100 mg/kg on day 5 plus *nab*-paclitaxel 30 mg/kg/d IV for 5 consecutive days. Animals were killed and tumors were harvested 1 hour after the last gemcitabine dose. Gemcitabine concentrations in tumors were measured in the JH Analytic Pharmacology Core. Briefly, tumor tissue homogenates were prepared. After liquid extraction and evaporation of homogenates, the sample was dissolved in 100 μL of methanol/water (10:90, volume/volume). The analytes were separated on a YMC Jsphr M80TM C18 column (Waters, Millford, MA), and gemcitabine and dFdU (a gemcitabine metabolite) were monitored by tandem mass spectrometry.

RESULTS

Patients

A total of 67 patients were enrolled and evaluated (Table 1). All patients have discontinued therapy either because of progressive disease (48%), unacceptable toxicity without progressive disease (18%), patient discretion (17%), investigator discretion (8%), AE (8%), or other (2%). The most common treatment-related AEs that led to treatment discontinuation were neuropathy and fatigue.

MTD and DLTs

Of the first six patients treated at dose level 1 (100 mg/m^2 *nab*-paclitaxel cohort), two patients had their day 8 treatment held: one patient with a possible history of ethanol abuse had asymptomatic neutropenia (absolute neutrophil count 0.85 $\times$ 10^9 cells/L), and a 79-year-old patient had asymptomatic thrombocytopenia (platelet count 60 $\times$ 10^9 cells/L). In three of those first six patients, radiologic responses were observed. Because of the confounding factors in two patients with dose delays, the potentially promising level of antitumor activity with this regimen, and the excellent tolerability in the remaining patients, the protocol was modified to allow for a total of 20 patients at dose level 1 rather than considering this dose level as having exceeded the MTD. Subsequently, dose escalation proceeded to dose level 2 and then 3. Of the three patients at dose level 3 (150 mg/m^2 of *nab*-paclitaxel), one patient died as a result of treatment-related systemic infection (neutropenia in the presence of a biliary stent) during cycle 1, and the MTD was established at dose level 2 (125 mg/m^2 of *nab*-paclitaxel). The other two patients at dose level 3 had grade 3 AEs that were resolved (leukopenia, fatigue, and neutropenia). A total of 44 patients were enrolled at dose level 2.

Table 1. Baseline Patient Demographics and Clinical Characteristics

Characteristic	*nab*-Paclitaxel mg/m^2 100 (n = 20) No.	%	125 (n = 44) No.	%	150 (n = 3) No.	%
Age, years						
Median	62		61		69	
Range	30-86		28-78		53-72	
Female sex	9	45	25	57	1	33
ECOG						
0	9	45	22	50	2	67
1	11	55	22	50	1	33
Site of metastatic disease						
Abdomen/peritoneal*	16	80	38	86	2	67
Liver	11	55	34	77	2	67
Liver only	1	5	2	5	1	33
Lung	5	25	18	41	1	33
Lung only	1	5	5	11	1	33
Other	10	50	12	27	1	33
No. of metastatic sites						
1	6	30	8	18	1	33
2	8	40	18	41	2	67
≥ 3	6	30	18	41	0	
CA19-9 baseline levels, n	15		37		2	
Normal†	2	13	6	16	1	50
Elevated	13	87	31	84	1	50
CA19-9 baseline, μ/mL						
Median	1,148		881		181	
Range	14-180,062		1-96,990		23-339	
Previous treatment						
Prior chemotherapy‡	3	15	10	23	1	33
Prior adjuvant therapy	3	15	10	23	1	33
With gemcitabine	1	5	5	11	0	
With capecitabine	1	5	4	9	0	
With FU	2	10	1	2	0	
With docetaxel	0		2	5	0	
With erlotinib	0		0		1	33
Time since adjuvant therapy,§ months					5	
Median	64		12			
Range	9-81		1-23			

Abbreviations: ECOG, Eastern Cooperative Oncology Group; FU, fluorouracil; *nab*, albumin bound.
*Peritoneal was not collected separately.
†Cutoff for normal range was < 37 u/mL. Approximately 10% to 15% of patients with pancreatic cancer lack Lewis antigens and thus lack the ability to secrete CA19-9.
‡There were no prior neoadjuvant therapy regimens.
§Time from last dose of prior adjuvant therapy to metastatic disease.

Efficacy Results

Survival. In patients treated at the MTD of 125 mg/m^2 of *nab*-paclitaxel (n = 44), the median PFS was 7.9 months (95% CI, 5.8 to 11.0 months), median OS was 12.2 months (95% CI, 8.9 to 17.9 months; Fig 1A), and the 1-year survival was 48%. For all 67 patients, median PFS was 7.1 months (95% CI, 5.7 to 8.0 months), with median OS of 10.3 months (95% CI, 8.4 to 13.6).

Response rate. The overall response rate (ORR) was 46% for all patients (N = 67). In the 100 (n = 20) and 125 (n = 44) mg/m^2 *nab*-paclitaxel cohorts, the response rates were 45% and 48%, respectively (Table 2). The overall disease control rate was 60% and 68%, respectively.

PET scan analysis. FDG PET scans were available for 55 patients. The median decrease in metabolic activity was 79% for all three cohorts together at 12 weeks. In the 125 mg/m^2 *nab*-paclitaxel cohort (n = 38), the reduction in FDG uptake was greater compared with the 100 mg/m^2 cohort (n = 14; 68% *v* 53%; P = .044) at 6 weeks, but not at 12 weeks (74% *v* 76%; P = .13, respectively). When PET analyses from all three cohorts were combined, patients with a complete metabolic response, defined according to the European Organisation for Research and Treatment of Cancer criteria by the absence of FDG uptake, had a significantly improved OS compared with patients without a complete metabolic response (median 20.1 *v* 10.3 months, respectively; P = .01; Fig 1B).

Treatment Exposure

Across all *nab*-paclitaxel doses, patients received 81% of the planned dose and 85% of the planned gemcitabine dose. The median number of cycles administered was 6.0 (range, 1 to 24) for all patients. Twenty-five percent of patients had a *nab*-paclitaxel dose reduction, with 20% in the 125 mg/m^2 cohort. Thirty-one percent of patients had a gemcitabine dose reduction, with 43% in the 125 mg/m^2 cohort. For all patients and in the 125 mg/m^2 cohort, 72% and 70% of patients had a *nab*-paclitaxel dose delayed, respectively, mainly due to AEs. For all patients, 73% patients had a dose delay of gemcitabine sometime in their treatment, mainly because of AEs.

Safety Results

The DLTs were sepsis and neutropenia. The most common treatment-related AEs of any grade were anemia (98%), leukopenia (91%), neutropenia (89%), thrombocytopenia (83%), fatigue (76%),

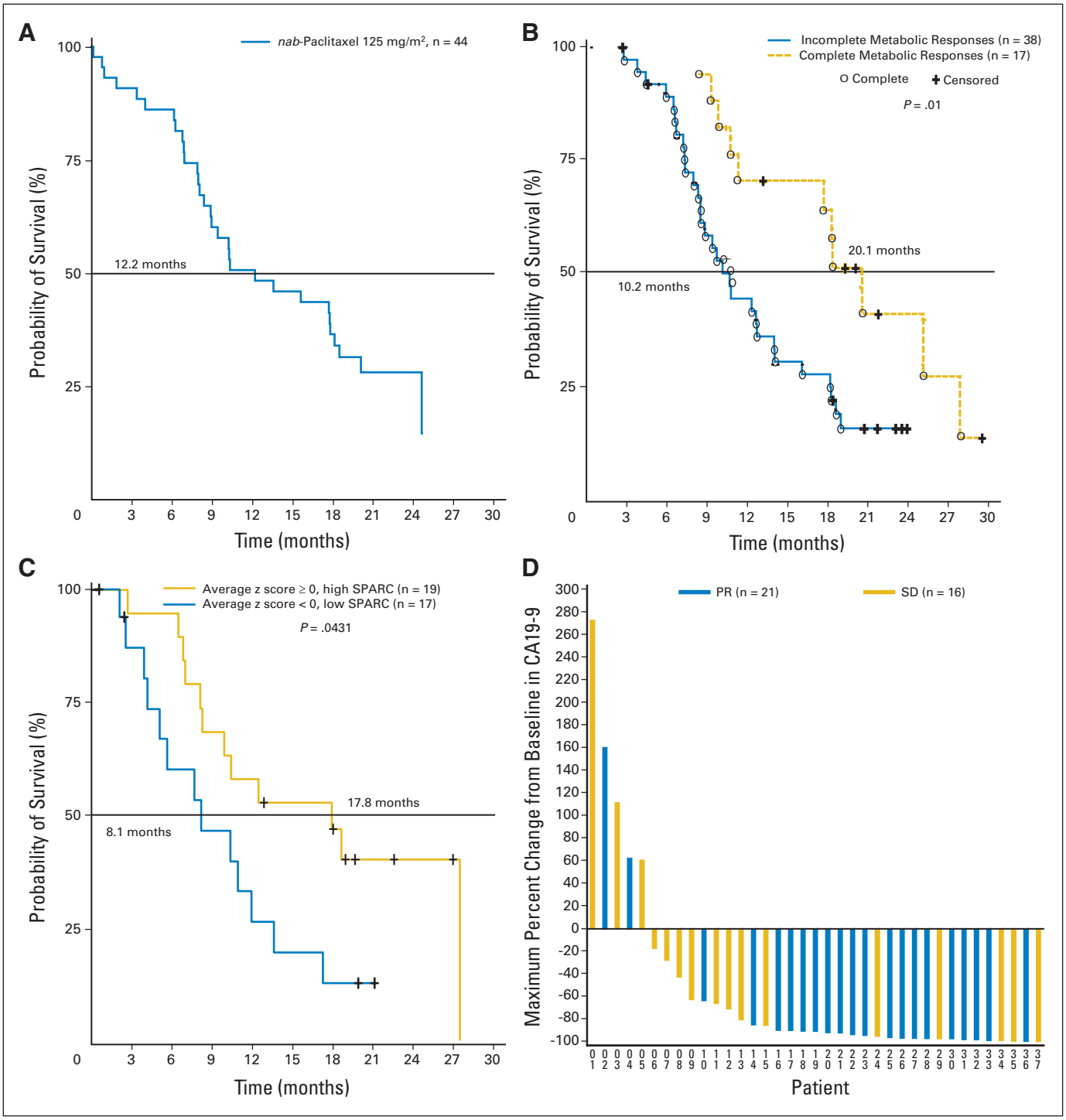

Fig 1. (A) Median overall survival in patients receiving 125 mg/m^2 of albumin-bound (*nab*) paclitaxel followed by 1,000 mg/m^2 of gemcitabine. (B) Median overall survival correlated with a complete metabolic response compared with baseline, defined according to the European Organisation for Research and Treatment of Cancer criteria by the absence of [^{18}F]fluorodeoxyglucose uptake (cohorts 1 and 2). (C) Median survival correlated with secreted protein acidic and rich in cysteine (SPARC; all cohorts). (D) Maximum percentage change in CA19-9 levels in patients receiving 125 mg/m^2 of *nab*-paclitaxel followed by 1,000 mg/m^2 of gemcitabine. PR, partial response; SD, stable disease.

alopecia (76%), sensory neuropathy (63%), and nausea (48%). Most of these treatment-related AEs were grade 1 and 2 (Table 3). Specifically, the most common grade ≥ 3 *nab*-paclitaxel–related nonhematologic AEs were fatigue (21%) and sensory neuropathy (15%). Of the grade ≥ 3 treatment-related hematologic AEs, neutropenia (67%), leukopenia (44%), and thrombocytopenia (23%) were the most common.

Biomarkers

SPARC. SPARC status was evaluated in 36 patients. Applying the average z-score algorithm to all 42 variables, patients were classified into high-SPARC (average z-scores ≥ 0, n = 19) and low-SPARC groups (average z-scores < 0, n = 17). A significant increase in OS was observed for patients in the high-SPARC group compared with patients in

Table 2. Response Rates, Disease Progression, and Disease Control Rates for All Patients and in the 125 mg/m^2 *nab*-Paclitaxel Cohort

Response Result	Dose Level 2 (n = 44) No.	Dose Level 2 (n = 44) %	All Dose Levels (n = 67) No.	All Dose Levels (n = 67) %
Complete response	0		3	4
Partial response	21	48	28	42
Stable disease*	9	20	12	18
Progressive disease	7	16	15	22
Disease control rate†	30	68	43	64

*Stable disease was defined as ≥ 16 weeks.
†Disease control rate was defined as the percentage of patients with complete and partial response and stable disease ≥ 16 weeks.

the low-SPARC group (median OS, 17.8 *v* 8.1 months, respectively; $P = .0431$; Fig 1C). Furthermore, SPARC level remained a significant predictor for the OS in a multivariate Cox regression model after adjusting for clinical covariates, including sex, race, age, treatment, and baseline CA19-9 level ($P = .041$). Additionally, stromal SPARC was significantly correlated with OS ($P = .013$), but not SPARC in tumor cells ($P = .15$).

CA19-9 levels. Rapid decreases in CA19-9 levels were observed, with the median time to maximum decrease of 89 days. In the 125 mg/m^2 cohort, 92% evaluable patients (34 of 37) had a ≥ 20% decrease in CA19-9, 78% (29 of 37) had a ≥ 50% decrease, and 70% (26 of 37) had a ≥ 70% decrease in CA19-9. The median maximum percentage change in CA19-9 level was 91% for all patients and also for patients in the 125 mg/m^2 cohort (Fig 1D). CA19-9 levels were correlated with increased survival. Patients with ≥ 50% decrease in CA19-9 levels had a 62% ORR and 8.0 and 13.6 median months of PFS and OS, respectively, whereas those with less than 50% decrease in CA19-9 level had a 33% ORR and 3.6 and 6.5 months of PFS and OS, respectively ($P = .105$, $< .001$, and .004 for ORR, PFS, and OS, respectively).

Preclinical Study Results

Gemcitabine and *nab*-paclitaxel alone resulted in tumor regressions in two (18%) and four (36%) of 11 patient-derived xenografts, respectively. However, gemcitabine plus *nab*-paclitaxel chemotherapy resulted in tumor regressions in seven (64%) of 11 cases. The aggregate tumor regression response in individual xenografts derived from the 11 parental cases were 22 (24%) of 90, 34 (36%) of 95, and 53 (55%) of 96 for gemcitabine, *nab*-paclitaxel, and gemcitabine plus *nab*-paclitaxel, respectively (Fig 2A).

We analyzed the stromal content of two gemcitabine-resistant tumors in each of the treatment groups. Mice treated with vehicle or gemcitabine exhibited a profuse desmoplastic stroma, as demonstrated by the collagen type1 fibers (Fig 2B). In contrast, *nab*-paclitaxel treatment depleted the desmoplastic stroma as evidenced by compact "back-to-back" arrangement of neoplastic glands separated by "wisps" of collagen. The reduction in stromal content was accompanied by dilated blood vessels in the tumor milieu, which were particularly prominent in the combination therapy cohort. An approximately three-fold increase in mNestin, marker of endothelial cells, was observed in xenografts receiving combination therapy as compared with control tumors, consistent with increased stromal endothelial cell content. The reduction in tumor stroma and the accompanied increase in vascularization facilitated the delivery of gemcitabine to these tumors.

The intratumor concentration of gemcitabine increased by 2.8-fold in the gemcitabine plus *nab*-paclitaxel treated tumors compared with gemcitabine-alone treated mice (Fig 2C).

Table 3. Selected Treatment-Related Adverse Events

Adverse Events	Dose Level 1 (n = 20) No.	Dose Level 1 (n = 20) %	Dose Level 2 (n = 44) No.	Dose Level 2 (n = 44) %	Dose Level 3 (n = 3) No.	Dose Level 3 (n = 3) %
Nonhematologic events						
Diarrhea						
Grade 1	1	5	7	16	1	33
Grade 2	1	5	6	14	0	
Grade 3	3	15	1	2	0	
Grade 4	0		0		0	
Fatigue						
Grade 1	4	20	10	23	0	
Grade 2	9	45	13	30	1	33
Grade 3	1	5	12	27	1	33
Grade 4	0		0		0	
Nausea						
Grade 1	7	35	11	25	1	33
Grade 2	2	10	9	20	1	33
Grade 3	0		1	2	0	
Grade 4	0		0		0	
Sensory neuropathy						
Grade 1	5	25	15	34	0	
Grade 2	1	5	9	20	2	67
Grade 3	1	5	9	20	0	
Grade 4	0		0		0	
Vomiting						
Grade 1	1	5	10	23	1	33
Grade 2	2	10	3	7	1	33
Grade 3	0		3	7	0	
Grade 4	0		0		0	
Hematologic events						
Anemia						
Grade 1	7	35	10	23	2	67
Grade 2	11	55	27	63	1	33
Grade 3	1	5	6	14	0	
Grade 4	0		0		0	
Leukopenia						
Grade 1	2	10	6	14	1	33
Grade 2	12	60	9	21	1	33
Grade 3	4	20	16	37	1	33
Grade 4	0		8	19	0	
Neutropenia						
Grade 1	4	20	6	14	0	
Grade 2	3	15	1	2	1	33
Grade 3	8	40	11	26	2	67
Grade 4	2	10	21	49	0	
Febrile neutropenia						
Grade 1	0		0		0	
Grade 2	0		0		0	
Grade 3	1	5	1	2	0	
Grade 4	1	5	0		0	
Thrombocytopenia						
Grade 1	5	25	18	42	2	67
Grade 2	5	25	9	21	1	33
Grade 3	2	10	8	19	0	
Grade 4	1	5	4	9	0	

NOTE. One patient at dose level 3 had grade 5 sepsis.

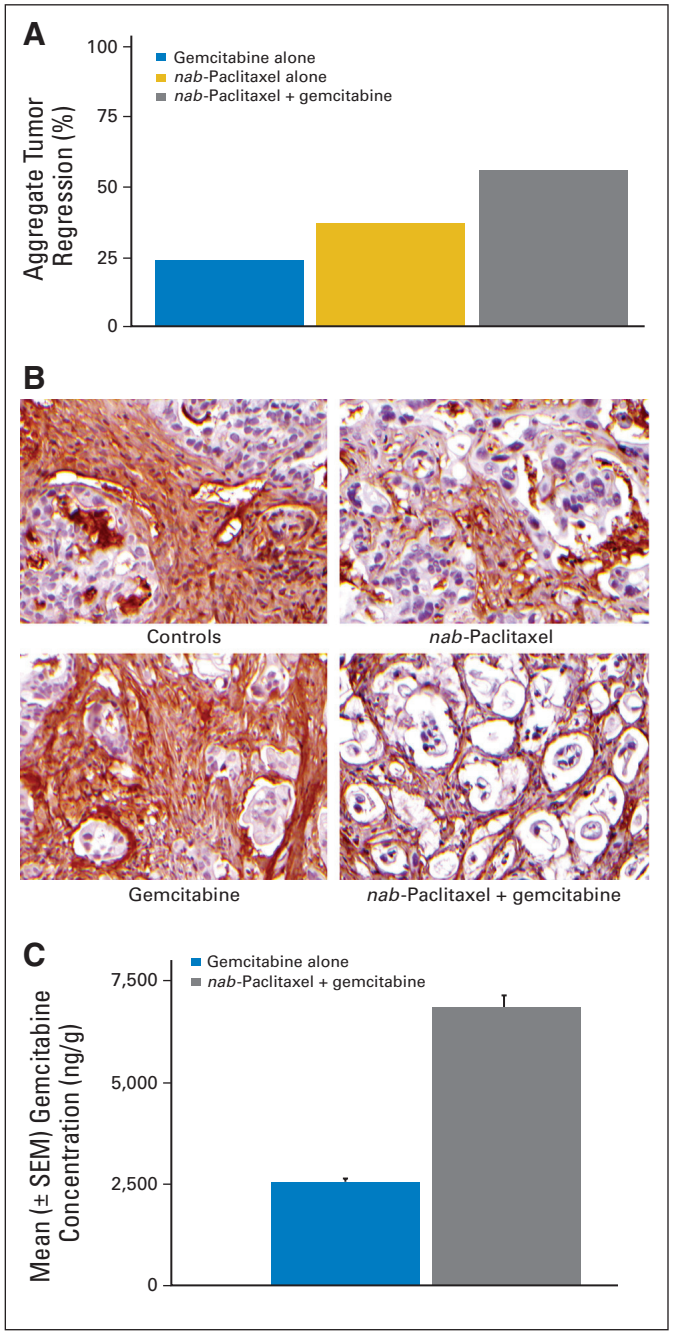

Fig 2. (A) Percentage incidence of aggregate tumor regression in response to gemcitabine, albumin-bound (*nab*) paclitaxel, and gemcitabine plus *nab*-paclitaxel in individual xenografts derived from the 11 parental cases. (B) Immunohistochemical assay for collagen type 1 fibers in a gemcitabine-resistant human pancreatic cancer xenograft treated with *nab*-paclitaxel, gemcitabine, or gemcitabine plus *nab*-paclitaxel. (C) Intratumor concentration of gemcitabine in human pancreatic cancer xenografts.

DISCUSSION

The MTD (the recommended dose for phase III) was 1,000 mg/m^2 of gemcitabine plus 125 mg/m^2 of *nab*-paclitaxel administered weekly for 3 weeks, repeated every 4 weeks. The 48% ORR, 12.2 months of OS, and 1-year survival of 48% at the MTD is among the highest reported for a phase II study in patients with PDA, including the fluorouracil, leucovorin, irinotecan, and oxaliplatin regimen,[26] which in a recent randomized phase III trial produced significantly improved survival compared with gemcitabine alone.[27] Additionally, this current study is among the first to formally assess PET scan responses in pancreatic cancer. Results showed that a complete loss of FDG metabolic activity was associated with favorable survival. In accordance with published results showing that CA19-9 is a prognostic marker for both PFS and OS,[28] decrease from baseline CA19-9 in the present study was an independent prognostic factor for OS. Overall, SPARC expression was not correlated with baseline CA19-9 levels, indicating that SPARC is a predictive marker independent of CA19-9 levels. Although an increase in SPARC level was correlated with improved OS, the significant increase was specific to elevated stromal SPARC and not SPARC in tumor cells. This is particularly important because historically, SPARC expression in the stroma, but not in the tumor, has been associated with poor survival,[29,30] suggesting that a unique mechanism of action of the present regimen may play a role in this reverse outcome. Together these observations indicate that stromal SPARC expression may be an important marker of early activity of gemcitabine plus *nab*-paclitaxel combination regimens in advanced pancreatic cancer.

The preclinical studies were subsequently initiated on the basis of the encouraging responses seen in the clinical trial. In the present preclinical study, *nab*-paclitaxel alone and in combination with gemcitabine depleted the peritumoral desmoplastic stroma, and intratumoral concentration of gemcitabine increased in mice treated with *nab*-paclitaxel versus those receiving gemcitabine alone. We speculate that reducing the dense tumor stroma, a histologic hallmark of PDA, may allow the chemotherapeutics to reach the tumor tissue more efficiently. Although these preclinical results were compelling in the athymic mouse, it has been noted that the mouse stromal cells may be transformed in the presence of human xenograft.[31,32] Other existing models of PDA (eg, *Kras* mutations that harbor similar precancerous lesions as humans) may be needed to confirm the stromal depletion seen in this model. The stromal depletion and the increased survival with SPARC expression observed in this study indicate that, in addition to intrinsic antitumor effects against the cancer cell, *nab*-paclitaxel may target stromal SPARC and facilitate delivery of chemotherapy. These data are consistent with a recent preclinical study targeting the hedgehog pathway in pancreatic cancer[23] and suggest that stroma-directed treatments may be a new treatment strategy. In particular, the antitumor activity of gemcitabine plus *nab*-paclitaxel combination therapy may, in part, be explained by the use of the albumin receptor(gp60)–caveolin-1–caveolae–SPARC pathway to increase intratumoral drug concentrations.[33]

Although the results of this clinical phase I/II study are promising, as with any nonrandomized study, patient selection may have influenced the outcome, and validation by a larger randomized trial is necessary. Given the favorable safety profile and the encouraging antitumor activity of the *nab*-paclitaxel plus gemcitabine regimen, a phase III study comparing gemcitabine plus *nab*-paclitaxel and gemcitabine alone has been initiated.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked

with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Neil Desai, Celgene (C); Vuong Trieu, Celgene (C); Jose L. Iglesias, Celgene (C); Hui Zhang, Celgene (C); Patrick Soon-Shiong, Abraxis BioScience (C); Tao Shi, Celgene (C) **Consultant or Advisory Role:** None **Stock Ownership:** Neil Desai, Celgene; Vuong Trieu, Celgene; Jose L. Iglesias, Celgene; Patrick Soon-Shiong, Celgene; Tao Shi, Celgene **Honoraria:** Ramesh K. Ramanathan, Abraxis BioScience **Research Funding:** Daniel D. Von Hoff, Celgene; Ramesh K. Ramanathan, Abraxis BioScience; Mitesh J. Borad, Abraxis Oncology; Daniel A. Laheru, Celgene; Lon S. Smith, Abraxis BioScience; Ronald L. Korn, Scottsdale Medical Imaging **Expert Testimony:** None **Other Remuneration:** None

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Manuscript writing: All authors
Final approval of manuscript: All authors

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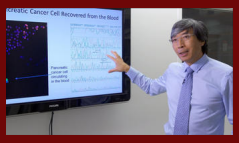
60 MINUTES
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DISRUPTING CANCER

Billionaire Dr. Patrick Soon-Shiong is turning heads with unconventional ways of treating the deadly disease

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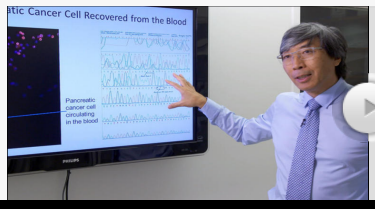
The following is a script from "Disrupting Cancer" which aired on Dec. 7, 2014. Dr. Sanjay Gupta is the correspondent. Draggan Mihailovich, producer.

Cancer has outwitted scientists and doctors for decades. More than 1,500 people still die of the disease every day in this country. But scientists will tell you they have learned more about cancer in the last five years than ever before. And no one is more optimistic about what that will mean for patients than Dr. Patrick Soon-Shiong. He's been called a genius, a showman, an innovator and a hypester. He's also the richest man in Los Angeles, a doctor and entrepreneur who is worth an estimated $11 billion.

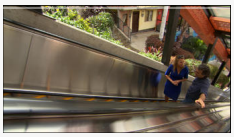


60 MINUTES OVERTIME

THE BILLIONAIRE SHAKING UP THE WORLD OF CANCER

RECENT SEGME


Lost


The New


Prime M
Netanya


The Spe
House

Give Dr. Patrick Soon-Shiong a white board and a few markers, and like a mad scientist he'll diagram how he thinks cancer can be beaten. He wants to attack on multiple fronts and is confident there is a pathway to the cure. For 45 minutes, he outlined his vision from beginning to end.

Dr. Patrick Soon-Shiong and Dr. Sanjay Gupta / **CBS NEWS**

Sanjay Gupta: This is a crazy looking board (laughter)...

Patrick Soon-Shiong: This is what goes on in my head you know, this is, it's like bursting. It just has to get this stuff out right?

Sanjay Gupta: Are we looking inside your head?

Patrick Soon-Shiong: Yeah, I think so, a little bit (laughter)...

Sanjay Gupta: How long before we get to here?

Patrick Soon-Shiong: I'm incredibly encouraged to say that we are on the path. And the technology to actually do all these things is not just hypothetical.

Technology is the main weapon Soon-Shiong is deploying against cancer. In October, at his company's headquarters in Los Angeles, final tests were being run on high-speed tumor genome sequencing machines that Soon-Shiong is convinced will unmask the molecular secrets to cancer.

Patrick Soon-Shiong: And for the first time with this technology we can watch it, catch it, outsmart it, and play chess at this multi-dimensional level.

To understand the significance of what Soon-Shiong is touting, it's important to know what cancer is.

Patrick Soon-Shiong: A cancer is not what people think, cells growing. Cancer is actually the inability of the cells to die.

The key is figuring out the genetic mutation or glitch that prevents cells from dying a natural death. Soon-Shiong's hope is to provide patients with the precise genetic mutations that fuel their cancer regardless of where tumors are found in the body.

Patrick Soon-Shiong: The mutation that happens in lung cancer could be the exact same

Sanjay Gupta: That's a big idea. I mean, you know, the idea that the breast cancer specialist, they're looking for breast cancer mutations and they may be missing the ball.

Patrick Soon-Shiong: Absolutely.

A lung cancer drug could work on breast cancer, for instance, if the mutation is the same.

The concept of doing away with labeling the disease by where it's found is not unique to Soon-Shiong, but it is a tectonic shift in the fight against cancer, the notion of classifying a cancer by its mutation.

Patrick Soon-Shiong: Imagine reclassifying cancer. And having people conceive and understand that cancer's a slew of rare diseases. So I am very excited because we are gonna create this revolution.

Sanjay Gupta: And what's it going to mean?

Patrick Soon-Shiong: Well, it's going to mean you have a better shot at having a better outcome and having a quality of life and actually turn the cancer hopefully into a chronic disease.

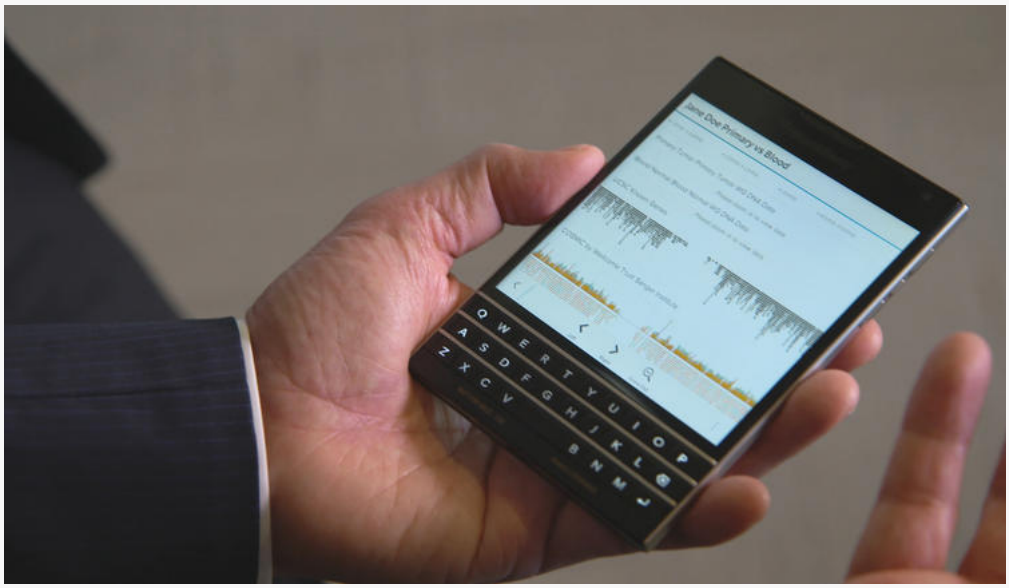
Sanjay Gupta: That's very optimistic. Realistic as well?

Patrick Soon-Shiong: I think so. Very much so.

Soon-Shiong has appointed himself to lead this revolution. Cancer genome sequencing is not new but what's different about Soon-Shiong's project is the scale. He has spent nearly a billion dollars of his own money to build a massive infrastructure, run by super computers, to find every single genetic mutation that could drive cancer.

This is Soon-Shiong's plan: A patient, anywhere in the world, has his tumor biopsied. The tumor cell's complete genetic map is then created all the way down to the proteins that are produced. What only recently took months can now be done in a day.

Ultimately, personalized information for each cancer patient would show up in the palm of his hand.



Patrick Soon-Shiong: This is the baby...

Sanjay Gupta: At the end of the day, someone has a tumor and they could find out the complete analysis of that tumor and what the perfect drug is to treat it?

Patrick Soon-Shiong: Correct. That's what's exciting. It's not the end of the day. This is what we think we can bring to the world now.

But some in the cancer world fear Soon-Shiong is getting ahead of himself, that he's declaring victory before any of this has been proven to work consistently.

Derek Raghavan: It's show me the money, show me the data. Show me that it's true.

Dr. Derek Raghavan, a renowned oncologist and researcher, is president of the Levine Cancer Institute in Charlotte, North Carolina.

60 MINUTES: SEGMENT EXTRAS

HOW TO LABEL CANCER

Sanjay Gupta: Dr. Soon-Shiong says, 'Look, if we can figure out which mutation's driving cancer, we're gonna be able to find the drugs that treat cancer.' Is that a fair theory?

Dr. Derek Raghavan: Yes. That's a fair theory. But to say I can throw a tumor into a gizmo, and that gizmo will tell me the answer in a few minutes, and everything will flow from that, I don't think we're there now. I don't think we'll be there next year. I think there's just too much hard, complex science that has to be done before this is state of the art. But it's a very cool idea for the future.

The vast majority of mutations are actually not a threat. So to figure out which mutations are dangerous, Soon-Shiong is going back in time.

Patrick Soon-Shiong: This national treasure...

Sanjay Gupta: Wow...

In the basement of the John Wayne Cancer Institute in Santa Monica, California, decades of cancer tissues were stored by scientists in deep freeze vats. Now Soon-Shiong wants to use technology that didn't exist back then to map the genomes of these thousands of tissues in order to look for critical patterns.

to death. And other mutations may not.

Patrick Soon-Shiong: That's exactly right. And ask the question, "Why did this patient live and why did this patient die? Why did this treatment work, why did that not work?"

To make any of this work, Soon-Shiong believes you need to upend the way cancer drugs are developed. He's started a biotech company to try to dramatically ramp up production.

Patrick Soon-Shiong: I know it sounds an audacious goal but you need to actually develop 20 to 30 drugs a year to actually get ahead of this game.

Sanjay Gupta: Right now it takes a few years to create a single drug and you're talking about 30 drugs in, in one year. Is that really feasible?

Patrick Soon-Shiong: I think that's where we have the challenge in pharmaceutical industry. We actually need to change the way we develop drugs now.

Soon-Shiong is impatient with the pace of drug approvals. In the early 90s, he invented a drug called Abraxane that treats pancreatic, lung and breast cancer patients. But more than a decade passed before the FDA approved it.

Patrick Soon-Shiong: The problem is for cancer, however, we don't have that time. You know, if you have pancreatic cancer, you have two months, if you have metastases throughout your body. The war against cancer is a war against time.

Soon-Shiong is also frustrated with what he calls the trial and error cycle of cancer care.

Patrick Soon-Shiong: The truth of the matter, we treat cancer today, we guess. We take what we call the average results, put it in you, see if it works. If it doesn't work, oops, we'll try another drug. If it does work, we stop the drug. When you look back 10 years from now, it's almost barbaric.

The 62-year-old native of South Africa can afford to be outspoken because of his immense wealth. He doesn't need to rely on the government or Big Pharma for funding. Soon-Shiong is certain what he terms the Dark Age of cancer treatment is nearly over, and the Enlightened Age is about to begin.

Sanjay Gupta: What will the average person note about the Enlightened Age versus the Dark Age?

Patrick Soon-Shiong: The treatment doesn't need to be painful. Metastasis doesn't need to be a death sentence. Cancer could be a chronic disease...and treated towards the cure.

While the oncology world may cringe when he boasts, as he's prone to do, patients see him differently.

David Roy: The established community doesn't like false hope. But if you have a terminal disease like I do, you want some hope.

David Roy was diagnosed two years ago with stage four, metastatic pancreatic cancer. He was given four and a half months to live and told to settle his affairs. He called Dr. Soon-Shiong, whom he had met on a plane years before. Soon-Shiong recommended a UCLA oncologist who devised an unusual therapy that combined Abraxane with other cancer drugs. Then Soon-Shiong had Roy's tumor genome sequenced. Based on those results, Roy is now taking part in a clinical trial involving another front in cancer treatment.

Patrick Soon-Shiong: That's the T-cell and that's the cancer cell.

It's called immunotherapy. Soon-Shiong is not the only one working on it, but he was

it up. There's the cancer cell. And...

Sanjay Gupta: That's amazing. So you're literally watching cancer cells die here?

Patrick Soon-Shiong: Correct.

Sanjay Gupta: If you find these T-cells and you're able to isolate them, is the idea then, you know they could do the job, you could come out and grow them, proliferate them, and put 'em back in the body.

Patrick Soon-Shiong: Exactly.

Even though it's been two years now since David Roy's original diagnosis, he's realistic about his chances of survival. But he's convinced Soon-Shiong and other scientists are on track to dramatically decrease cancer death rates in the not too distant future.

David Roy: I'm not sure that it'll happen fast enough for me, but I have every confidence that my children and grandchildren won't be concerned about the things that I'm concerned about. We are on the edge here, of going from the oil lamp to electricity. And it is going to happen.

Soon-Shiong's most provocative idea, though, centers on how cancers may become metastatic.

He believes chemotherapy works best when administered in frequent, low doses and that in some cancers the traditional method of blasting a tumor with heavy doses of chemotherapy may be actually be counterproductive - because it could induce cancer cells to escape the hostile environment, enter the bloodstream and find a new home.

Sanjay Gupta: It's on the move.

Patrick Soon-Shiong: It's on the move. And it's looking for another place to land.

Patrick Soon-Shiong: Circulating tumor cells in the blood is the new frontier. Those are the circulating tumor cells.

Sanjay Gupta: That's incredible.

If cancer spreads the likelihood of survival decreases dramatically. So before individual rogue cancer cells fan out and form new tumors, Soon-Shiong wants to detect them with what are known as liquid biopsies. A person's blood sample is put through this bio-chip that separates normal blood cells from heavier, circulating tumor cells. This is a view inside the bio-chip as the tumor cells are being funneled to the top.

Patrick Soon-Shiong: And if we can now monitor the cancer cell in the blood we then have a path to getting this and winning this war. We never had those paths before.

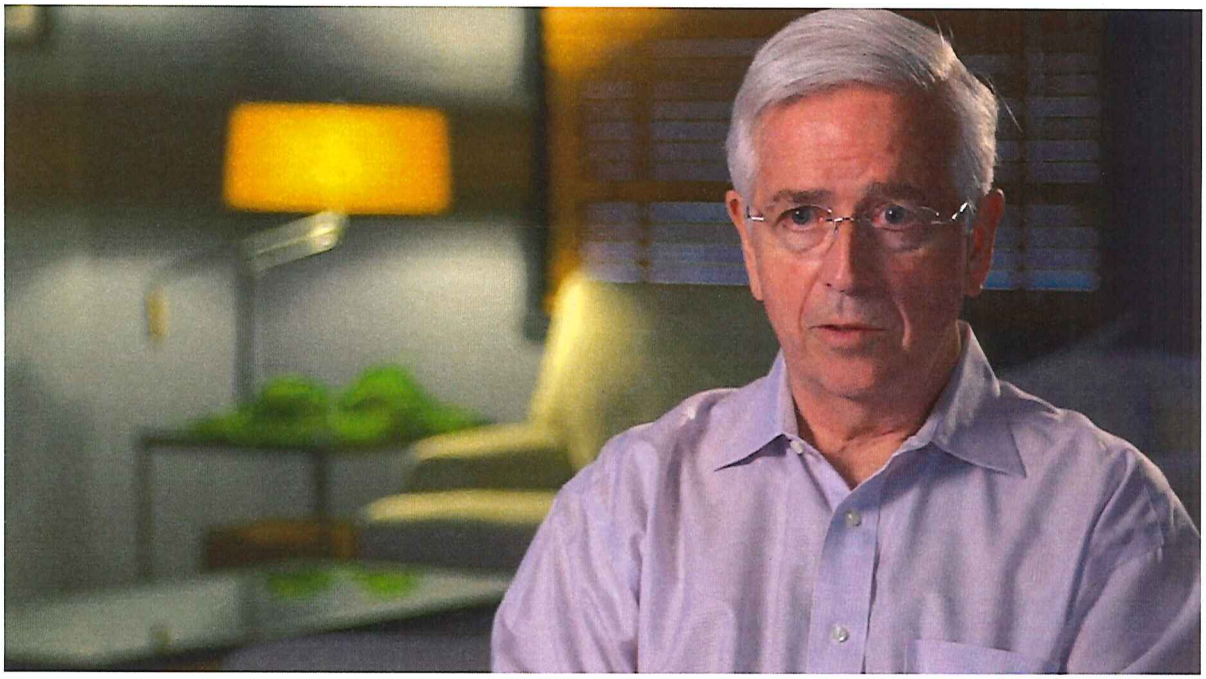
After pulling out the circulating tumor cells, scientists can take them back to the genome sequencer to look for new mutations that made them resistant to the initial treatment...and hopefully find a new drug to treat it. It's yet another angle Soon-Shiong is taking to disrupt cancer.

Sanjay Gupta: You got genomics. You have circulating tumor cell liquid biopsies. Death by T-cell. Why are you the one taking all this on, I mean these are lots of different types of things...

Patrick Soon-Shiong: You know, somebody once said to me, "You know, Patrick, you're all over the place." And I said, "You have to be all over the place" because I'm trying to fight this war from all over the place. Because you can't, there's no one single magic bullet.

Exhibit 17

David Roy Diagnosed with Stage 4 Metastatic Pancreatic Cancer



View Video:
https://nantworks.box.com/v/david-roy

Exhibit 18

Diane Diagnosed with Pancreatic Cancer

View Video:
https://nantworks.box.com/v/diane-pancreatic